Oxidation of Ethyl Hexadec-1-enyl Ether, A Plasmalogen Model, in the Presence of Unsaturated Esters[1]

To test the susceptibility of plasmalogen lipids to oxidation, a series of experiments was run on the model compound ethyl hexadec-1-enyl ether, which was prepared from hexadecanol by a multistep synthesis. Oxidation experiments were carried out on neat mixtures of the model ether with a variety of fatty esters in sealed vials under air. Disappearance of the ether, the fatty esters and, in one set of experiments, oxygen, was followed by gas chromatography. Ethyl stearate, which was inert to oxidation under the experimental conditions, served as internal standard. In the absence of polyunsaturate (linoleate or linolenate), alk-1-enyl ether underwent slow but measurable oxidation. In the presence of polyunsaturate, however, disappearance of the ether was greatly accelerated and proceeded at a rate comparable to that of the polyunsaturate. Reactions did not proceed in the absence of oxygen and were inhibited by antioxidant. The results suggest that oxidation of the alk-1-enyl ether functionality of plasmalogens should not be ignored as a factor that contributes to the oxidative instability of animal tissue or the development of rancidity in meat products.
Lipids 21, 648–651 (1986).

Plasmalogen phospholipids are found in high concentration among the membrane lipids of meat. For example, 20–30% of the choline glycerophosphatides and over 60% of the ethanolamine glycerophosphatides of bovine muscle are plasmalogen (1). Studies on lipid oxidation in foods typically focus on the reactivity of the polyunsaturated acyl functionality (2–5), but the presence of the alk-1-enyl ether functionality of plasmalogens has been ignored as a substrate for such oxidation. Nevertheless, plasmalogen oxidation has been cited in past physiological studies. In rat brain homogenates, for example, plasmalogens were found to undergo Fe^{2+}/ascorbate-catalyzed oxidation at the double bond site to yield, the authors speculated, an α,β-diol that further degraded to α-hydroxyaldehyde and (n-1)-aldehyde (6,7). Plasmalogens in erythrocytes were found to be prone to peroxidation catalyzed by glucose oxidase-glucose or dialuric acid (8). Plasmalogens in spermatozoa were found to be susceptible to oxidation under aerobic conditions (9). Curiously, studies that implicated lipid peroxidation as a mechanism of injury in cardiac tissue failed to consider plasmalogen oxidation (10–13), despite the abnormally high concentrations of this lipid class in cardiac tissue (14). Other studies have shown that the double bonds of alk-1-enyl ethers are subject to attack by singlet oxygen (15–17). One study focused on alkyl ether glycerols, which even without any unsaturation are prone to autoxidation (18). The present study was done to investigate whether plasmalogens, as represented by a model long chain alk-1-enyl ether, are susceptible to autoxidation under conditions that lead to such oxidation of long chain polyunsaturated esters.

EXPERIMENTAL

Synthesis of hexadecanal diethylacetal. Hexadecanal was synthesized from hexadecanol (Aldrich Chemical Co., Milwaukee, Wisconsin) by oxidation using dimethylsulfoxide (DMSO) and dicyclohexylcarbodiimide (Sigma Chemical Co., St. Louis, Missouri), according to the method of Fenselau and Moffatt (19). The crude aldehyde was converted to its diethylacetal using ethanolic benzene (NOTE: benzene is toxic and must be used with care and proper ventilation) with methanesulfonic acid according to the method of Gigg and Gigg (20). The acetal was purified by column chromatography using neutral alumina (80–200 mesh, Fisher Scientific Co., Fairlawn, New Jersey) and eluting the acetal with ether/petroleum ether (bp 30–60 C, 1:5, v/v). The column could be reused after eluting residual hexadecanol and hexadecanal with ether/methanol (1:1, v/v). The eluate stream was monitored by thin layer chromatographic (TLC) analysis.

Synthesis of ethyl hexadec-1-enyl ether. The alk-1-enyl ether was prepared from hexadecanal diethylacetal by preparing the chloroacetal with phosphorus pentachloride according to the method of Chebyshev et al. (21) and then inducing elimination of hydrogen chloride from the unisolated chloroacetal with triethylamine according to the method of Gigg and Gigg (20). Triethylamine hydrochloride was removed by passage through a small column of silica gel according to the Gigg and Gigg method, but with dichloromethane instead of ether. Purification of alk-1-enyl ether product was accomplished by column chromatography using silica gel (grade 60, 230–400 mesh, Aldrich). Elution, by hexane/benzene (9:1, v/v), was monitored by TLC. *Cis/trans* ratios were determined by gas chromatographic (GC) analysis (Fig. 1). The synthesized mixture of isomers (typically 70–80% *cis*) was used in subsequent oxidation studies. Separation of isomers could be achieved on silica gel by use of medium pressure liquid chromatography (Michel-Miller apparatus, Ace Glass Co., Vineland, New Jersey) with monitoring of the eluate by differential refractive index detection (Model R-401, Millipore/Waters Chromatography Division, Milford, Massachusetts). The *cis* isomer eluted before the *trans* (infrared λ_{max} 929 cm^{-1}), with some degree of overlap between the two species.

Commercial ethyl ether contains trace amounts of BHT antioxidant. Use of such ether in these syntheses will lead to product contaminated with BHT. Such product will show inhibition toward oxidation in subsequent experiments. BHT may be removed from any of these products by dissolving the material in hexane and extracting the solution with DMSO (22).

Ethyl esters of fatty acids. Ethyl esters were prepared from the corresponding methyl esters (methyl stearate and methyl linolenate, Nu-Chek Prep, Elysian, Minnesota;

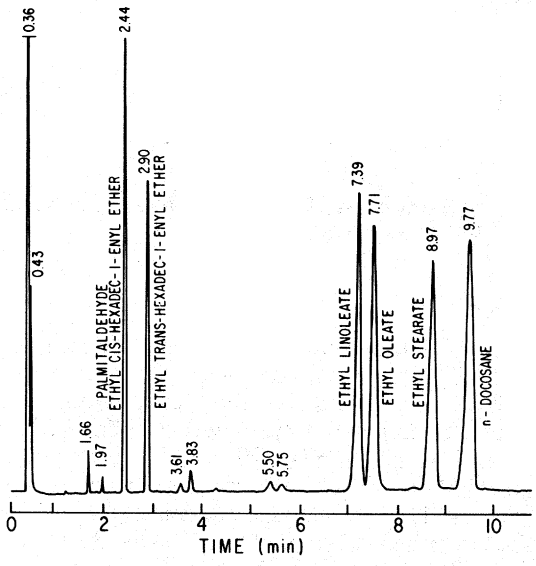

FIG. 1. GC trace of organic component separation. Conditions: 155 C isothermal, 2 mg/ml each component in isooctane; 0.5 μl injection, split ratio 100:1; capillary column, OV-101 bonded phase on fused silica. See Experimental section for further details.

TABLE 1

Composition of Reaction Mixtures Prior to Oxidation, in μmol

Component	Formula wt	Run 1	Run 2	Run 3	Run 4	Run 5
Cis-ether	268.5	9.9		2.4	2.2	2.3
Trans-ether	268.5	2.5		.97	.92	1.0
n-Docosane	310.6	7.1				
18:0	312.5	7.6	2.0	1.8	2.0	1.9
18:1	310.5	7.1				
18:2	308.5	7.4	1.8		1.9	
Oxygen	32.0	80	1100	1100	1100	1100
Vial volume, ml		9	120	120	120	120

methyl oleate, Applied Science, State College, Pennsylvania; methyl linoleate, Hormel Institute, Austin, Minnesota) by rapid transesterification at ambient temperature using ethanolic KOH (23).

Oxidation procedure. Quantitative mixtures of hexadec-1-enyl ether and appropriate ethyl esters, in amounts listed in Table 1, were prepared in 500 μl pentane. Aliquots of 10 μl then were transferred to each of nine "100-ml" capacity (actual capacity 120 ml) serum vials (Supelco, Bellefonte, Pennsylvania) and the pentane was removed under vacuum at ambient temperature. The vacuum was broken with room air and the vials were securely sealed with crimpable aluminum caps containing PTFE-lined butyl rubber septa (Perkin-Elmer, Norwalk, Connecticut). For each study, a set of such sealed vials was immersed in a controlled-temperature water bath at 86 ± 1 C. Vials then were withdrawn at designated times and cooled to ambient temperature. Samples of 20 μl of headspace gas were withdrawn at this time from the vials of run 1 to determine oxygen consumption. For all runs, internal surfaces of the vials were washed with 1 ml injected isooctane and the resultant solution was sampled for GC analysis.

Aldehyde generation from oxidized mixture. Aliquots of 500 μl of the above solution of reaction products from each of the set of vials from a repeat of run 4 (Table 1) were freed of solvent by evaporation under nitrogen and then subjected to mild hydrolysis by treatment with 15 mg ground Amberlyst 15 sulfonic acid resin (Rohm and Haas, Philadelphia, Pennsylvania) in 100 μl acetone and 0.15 μl water for 6 min at ambient temperature. The mixture was taken up in ether and filtered through glass wool. Amounts of hexadecanal so formed were determined by GC analysis.

TLC. Separation of fatty alcohol, aldehyde, diethylacetal and alk-1-enyl ether was achieved on plates of Silica Gel G by developing in toluene. Visualization of aldehyde could be accomplished by fuchsin-bisulfite spray; aldehyde so treated was detected as a purple-to-red spot. Acetal and alk-1-enyl ether spots were visualized in the same way after conversion to aldehyde by 1 min exposure to the vapors of concentrated hydrochloric acid. All species were visualized by spraying the plates with copper sulfate/phosphoric acid, followed by charring.

GC. Oxygen was determined on a Hewlett-Packard 7620A gas chromatograph using a stainless steel column, 6′ × 1/8″, packed with molecular sieve 13X. Determinations were run at ambient temperature using helium as carrier gas and thermal conductivity detection; samples of 20 μl were injected from a gas-tight locking syringe. Signal analysis was accomplished by routing the detector output to the integrating terminal of a Hewlett-Packard 5880A gas chromatograph.

Organic substrates were determined on a Perkin-Elmer Sigma-3 gas chromatograph using a fused silica wall-coated open-tubular column (Hewlett-Packard, Avondale, Pennsylvania) of 0.33 μ thick, cross-linked OV-101 methyl silicone, 12 m × 0.2 mm i.d. Determinations were made isothermally at 155 C using helium as carrier gas and a split ratio of 100:1. n-Docosane (Supelco) served as initial internal standard (run 1, Table 1), followed by ethyl stearate (18:0) once the latter was shown to be stable to oxidation under the experimental conditions. Signal analysis was accomplished in the same manner as described above. A GC trace that shows the separation of all components of interest is shown in Figure 1.

RESULTS AND DISCUSSION

Results are shown graphically (Figs. 2–5) for a series of runs at 86 C, a temperature that allowed for convenient measurement of substrate depletion. Data are graphed in normalized fashion to indicate the percentage of each remaining component at specified reaction times. Table 1 lists the amounts of starting materials for each run. Ethyl esters were selected over methyl esters to more closely parallel the ethyl alk-1-enyl ether. Oxygen content is estimated from the volume of a standard gas at standard temperature and pressure (22.4 l/mol) and the oxygen content of air (21%) (24).

Run 1 was carried out in a series of small vials (9-ml capacity) to allow a measurable depletion of oxygen. Results are depicted in Figure 2. Relative to the extent of oxidation over the 4.5-hr duration, oxygen consumption was minimal (18%). Further runs were done in much

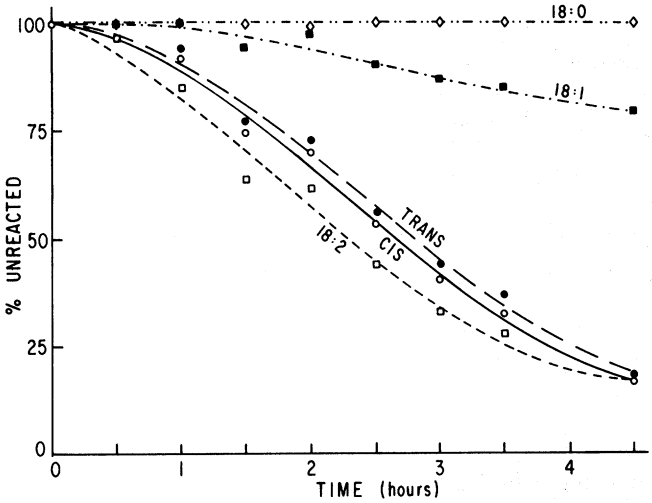

FIG. 2. Course of reaction at 86 C of run 1 (ethyl *cis*- and *trans*-hexadec-1-enyl ethers and 18:0, 18:1 and 18:2 esters; internal standard, n-docosane).

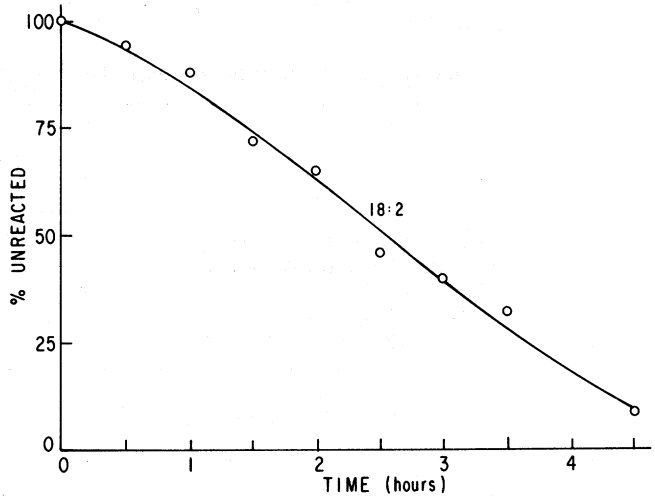

FIG. 3. Course of reaction at 86 C of run 2 (18:2; internal standard, 18:0).

larger vials (120-ml capacity) to eliminate oxygen content as a reaction variable. Run 1 also was carried out in the presence of n-docosane as internal standard to establish the inertness of 18:0. Later runs used 18:0 as internal standard. The results of this run show that 18:0 is stable under the conditions of oxidation and that ethyl oleate (18:1) reacts slowly. Depletion of ethyl linoleate (18:2) is considerable, and the isomers of alk-1-enyl ether are also highly reactive. The ethyl *cis*-hexadec-1-enyl ether (*cis*-ether), whose double bond configuration is the same as that of natural plasmalogen (25), is somewhat more reactive than the ethyl *trans*-hexadec-1-enyl ether (*trans*-ether).

Run 2 (Fig. 3) was carried out to demonstrate the autoxidation of 18:2 in the absence of alk-1-enyl ether. Run 3 (Fig. 4) was carried out analogously to investigate autoxidation of the alk-1-enyl ethers in the absence of polyunsaturate. Run 4 (Fig. 5) then was carried out on a combination of components of runs 2 and 3 to investigate the influence of polyunsaturate on alk-1-enyl ether oxidation. Finally, run 5 (Fig. 6) repeated conditions of run 4, but with ethyl linolenate (18:3) in place of 18:2. Runs 2 and 4 (Figs. 3 and 5) demonstrate that 18:2 autoxidation essentially is independent of the presence of alk-1-enyl ether, but, on the contrary, the extent of autoxidation of alk-1-enyl ether is dependent on the presence of 18:2 (runs 3 and 4, Figs. 4 and 5). Autoxidation of 18:3 is not unexpectedly faster than that of 18:2, and alk-1-enyl ether oxidation is accelerated by substitution of 18:2 and 18:3 (runs 4 and 5, Figs. 5 and 6).

The oxidative nature of the alk-1-enyl ether decomposition was established by repeating runs 1 and 3 with 0.1% antioxidant, w/w (BHA/BHT; Tennox 5; Kodak, Rochester, New York). The presence of antioxidant led to complete inhibition of decomposition over the 4.5-hr duration of the experiments. Moreover, reactions failed to proceed when nitrogen was substituted for air in the vial headspace. Thus, loss of alk-1-enyl ether cannot be attributed simply to hydrolysis by trace amounts of water.

To estimate what proportion of the oxidation of the alk-1-enyl ether occurred on the unsaturated side of the

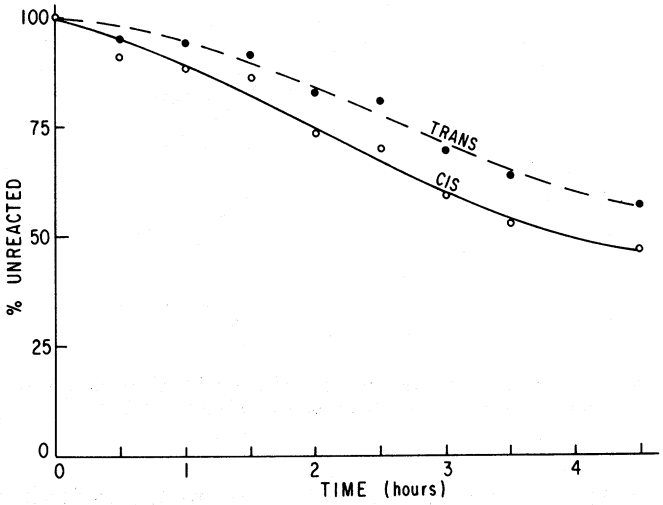

FIG. 4. Course of reaction at 86 C of run 3 (ethyl *cis*- and *trans*-hexadec-1-enyl ethers; internal standard, 18:0).

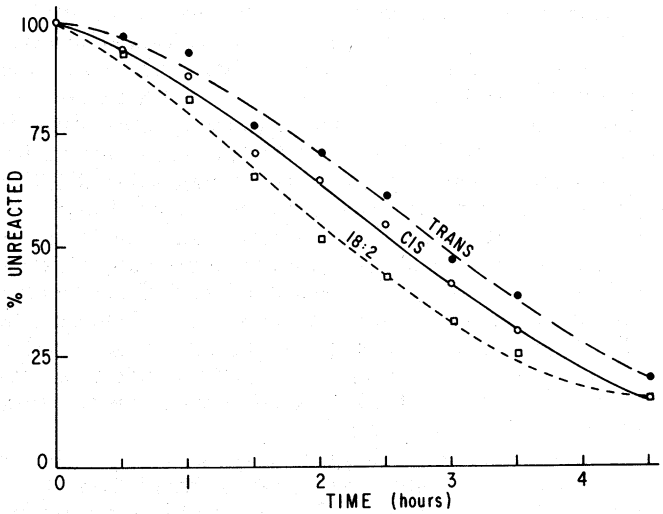

FIG. 5. Course of reaction at 86 C of run 4 (ethyl *cis*- and *trans*-hexadec-1-enyl ethers and 18:2; internal standard, 18:0).

TABLE 2

Hexadecanal Liberated from Aliquots of Run 4 by Hydrolysis

	Run time (hr)						
	0	1.0	1.5	2.0	3.0	3.5	4.5
Remaining total alkenyl ether (%)	100	82.5	54.7	52.1	37.5	21.2	19.8
Generated hexadecanal relative to 0 time (%)	100	89.0	68.4	73.5	53.4	36.1	40.2
Oxidized alkenyl ether oxidized on ethyl group[a] (%)	—	37[b]	30	45	25	19	25

[a]Average 30 ± 9%.

[b][(89.0 − 82.5)/(100 − 82.5)] × 100.

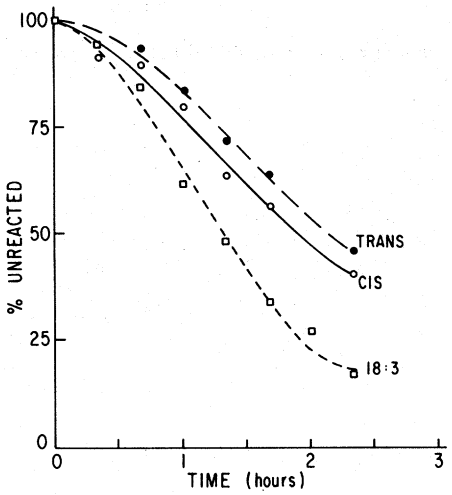

FIG. 6. Course of reaction at 86 C of run 5 (ethyl *cis*- and *trans*-hexadec-1-enyl ethers and 18:3; internal standard, 18:0).

molecule, run 4 was repeated. Following GC analysis of unreacted starting materials, aliquots of 100 μl from each vial of the set were acidified with Amberlyst 15 resin to liberate hexadecanal by hydrolysis. This aldehyde had been seen by GC analysis to be only a negligible product of oxidation (Fig. 1). The amount of hexadecanal liberated by hydrolysis was determined by GC analysis and was found to exceed the amount of remaining alk-1-enyl ether throughout the course of the reaction. Results, listed in Table 2, give insight on the extent of oxidation on the alk-1-enyl portion of the molecule, as opposed to the ethyl portion. The aldehyde is a hydrolysis product of the intact alk-1-enyl portion of the molecule, be it from the original ethyl hexadec-1-enyl ether or from an oxidation product that has the intact alk-1-enyl functionality. Results show that ca. 30% of the oxidation is not on the alk-1-enyl portion of the molecule. This demonstrates that both sides of the molecule are subject to oxidation, but that the unsaturated side is much more prone to oxidation than the saturated side.

These experiments suggest that the easily oxidized polyunsaturated esters accelerate autoxidation of the alk-1-enyl ether. This is highly significant because natural plasmalogens typically are also highly polyunsaturated (26). A future report will present results of more complex model plasmalogens to investigate whether the influence of polyunsaturation on alk-1-enyl ether oxidation might be an intramolecular process in the natural plasmalogen structure.

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